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Dextran protection of erythrocytes from low-pH-induced hemolysis

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Low-pH-induced hemolysis of erythrocytes is inhibited by dextrans. The protective effect was observed with dextrans larger than 40 kDa. Electron microscopy showed dextrans of 150 kDa in a tight association with the erythrocyte membrane. These results indicate that dextrans stop the low-pH-induced hemolysis by interacting with the acid-induced defects in the erythrocyte membrane [(1989) Biochim. Biophys. Acta, in press].

Erythrocyte hemolysis; Membrane defect, low-pH-induced; Dextran protection

1. INTRODUCTION

Dextrans interacting with red blood cells are adsorbed at the cell surface [1,2] and at high concentrations (about 40 mg/ml) induce the adhesion and rouleaux formation of erythrocytes [2,3]. Evidence was presented that dextrans penetrate between the carbohydrate groups on the red cell surface [4]. Dextran molecules also bind to the surface of phospholipid vesicles [5–7].

In recent studies we analyzed the low-pH-induced association of exogenous glycophorin and CD4 with erythrocyte membranes [8–10]. The association process was significant at pH values below 5. Under the same conditions, in the absence of proteins in the incubation media, hemolysis of the red blood cells occurred [10]. Evidence was presented that low pH induces defects in the red blood cell membrane in the absence of proteins in the incubation medium (presumably the sites of protein association). The defects appeared to develop in time into larger openings through which hemolysis occurred [10].

In the present study we show that dextran molecules can protect the erythrocyte membrane from low-pH-induced lysis. We present evidence of a close association of dextrans with the RBC membrane after incubation at low pH under protecting conditions. The protection process was characterized using two independent techniques: (i) spectrometric assay of the low-pH hemolysis and (ii) electron microscope visualization of the interaction between dextrans and the erythrocyte membrane.

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Abbreviations: au, arbitrary units; FD4, FD10, FD20, FD40, FD70 and FD150, fluorescein isothiocyanate labeled dextrans of average molecular masses 3.9, 9, 18.9, 40.5, 71.6 and 148.9 kDa, respectively; FITC, fluorescein isothiocyanate; PBS7.4, 5 mM sodium-phosphate buffer, 145 mM NaCl, pH 7.4; RBC, red blood cells

2. MATERIALS AND METHODS

2.1. *Chemicals*

Fluorescein isothiocyanate labeled dextrans of average molecular masses 3.9 kDa (FD4), 9 kDa (FD10), 18.9 kDa (FD20), 40.5 kDa (FD40), 71.6 kDa (FD70) and 148.9 kDa (FD150) were from Sigma (St. Louis, MO). Sodium cacodylate, glutaraldehyde and EMBED-812 were from Electron Microscopy Sciences (Ft Washington, PA); propylene oxide was from Polysciences (Warrington, PA) and Acrodisc filters were from Gelman Sciences (Ann Arbor, MI).

2.2. *Protection of low-pH hemolysis by dextrans*

1.5 ml of sodium acetate buffer, pH 4.6, 100 μl of dextran solution (2 mg dextran) and 30 μl of RBC suspension (approx. 1.5×10^7 cells) were mixed and incubated at 37°C for 1.5 min.

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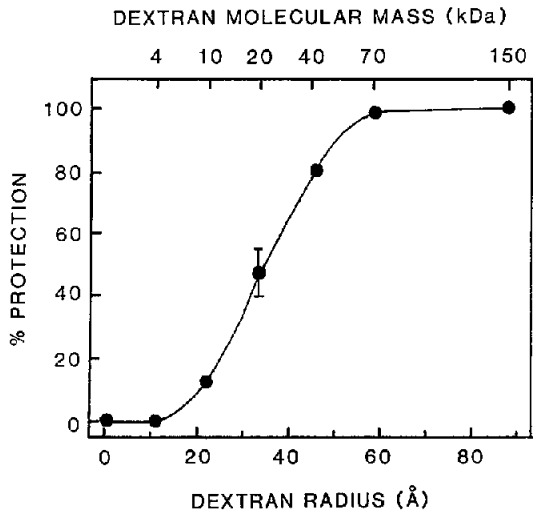

Fig.1. Protection against low-pH-induced hemolysis by dextrans. Erythrocytes were incubated at pH 4.6 with dextrans of different molecular masses for 1.5 min, 37°C. The percentages of protection against hemolysis were calculated from the hemoglobin content of the erythrocyte pellets after incubation using eqn 1 (see section 2). The values for average Stokes' radius of dextrans were obtained using the values determined by Peters [11,12].

After incubation, the RBC were washed twice by centrifugation (2 min, 3000 rpm, Eppendorf centrifuge). The resulting RBC pellets were suspended in 1.5 ml of PBS7.4 and hemolyzed by the addition of Triton X-100 (final concentration of Triton X-100 was 0.27%, v/v). The percentage protection from low-pH-induced hemolysis was calculated from absorbance measurements at 420 nm [10] using a Hitachi U-2000 spectrophotometer. The percentage of protection from hemolysis by a protecting agent was defined as:

$$\% \text{ protection} = [(A_p - A_o)/(A_{100} - A_o)] \times 100 \quad (1)$$

where A_p and A_o are the absorbances of the Triton X-100-treated pellets of RBC which were incubated at pH 4.6 with the protective agent (A_p) and without the protective agent (A_o), and A_{100} is the absorbance of the Triton X-100-treated pellet of RBC which were incubated in PBS7.4. A_{100} is defined as the A value for 100% protection.

2.3. *Preparation of samples for electron microscopy*

Erythrocytes were incubated with FD150 (1.5 min, pH 4.6, 37°C) and washed using the conditions described in section 2.2. The supernatant was removed from the final RBC pellet and 1.5 ml of ice-cold fixative solution (3% glutaraldehyde, 0.1 M sodium cacodylate, 0.03% $CaCl_2$, pH 7.4) was added to the pellet, which was not resuspended. Embedding, sectioning and poststaining were carried out as described in [10]. To observe low-pH-induced defects, the same experiment was carried out using sodium acetate buffer, pH 4.6, but in the absence of FD150.

3. RESULTS

3.1. *Protective effect of dextrans against low-pH hemolysis*

During short-time incubation at pH 4.6, erythrocytes began to hemolyse [10]. No hemolysis was observed if protein [9,10] or dextran was present in the reaction media. As presented in fig.1, protection against hemolysis was achieved with dextran molecules larger than 40 kDa. The high molecular mass dextrans, although at lower molar concentrations, were more effective in protecting the cells from hemolysis than the small dextrans. Using the value of Stokes' radius for dextrans determined by Peters [11,12], we estimated that efficient protection (>80%) against hemolysis is reached using dextrans with Stokes' radii greater than 45 Å (fig.1).

3.2. *Association of FD150 with defects in the RBC membrane during low-pH incubation*

Dextran particles in close association with the RBC membrane were seen in electron micrographs from thin sections of erythrocytes incubated at pH 4.6 for 1.5 min with FD150 in the incubation medium (fig.2a,b,c, arrows). Dextran particles were identified by irregularity of shape and electron opacity [13]. Defects in the red cell membrane [10] were observed when the same experiment was carried out in the absence of FD150 (fig.2d, arrows). These defects, observed in less than 10% of the cells, were the same as those described earlier [10], i.e., having average widths of 14 nm and depths of 12 nm. Smaller defects (average widths, 6 nm; depths, 11 nm) and patches of reduced density in the glycocalix were also observed. Defects were not observed when FD150 was present during the low-pH incubation.

4. DISCUSSION

Macromolecules like dextrans [14], albumin labeled with iodine 131, hemoglobin labeled with iron-59 [15] and ferritin [16] have been used to study hypotonic-induced pores in erythrocyte membranes. Dextrans were also shown to protect fibroblasts from T-cell-induced lysis [17] as well as erythrocytes from antibody-induced complement lesion [18] and from hemolysin-induced hemolysis [19].

In this study we show that dextran molecules inhibit the low-pH-induced hemolysis of human erythrocytes. Dextrans may stop the development of low-pH-induced small defects into hemolytic holes since there is evidence that the dextran molecules penetrate between the carbohydrate groups on the red cell surface [4] and that they interact with phospholipid membranes [5–7]. The interaction of dextrans with phospholipid head groups [5] and carbohydrate groups [4] should result in a 'coating' of the red cell surface with dextrans [20,21]. Thus, the mechanism of dextran protection may be the interaction with incipient defects which is facilitated by the close proximity of dextrans to defect sites. Our electron microscope observations support this explanation since ultrastructural analysis of red blood cells after incubation at pH 4.6 in the presence of FD150 showed dextran particles in tight association with the erythrocyte membrane (fig.2). If pores are forming during the low-pH incubation, dextrans which are too large to pass the transmembrane pores may also counteract the colloid-osmotic pressure of intracellular hemoglobin. The func-

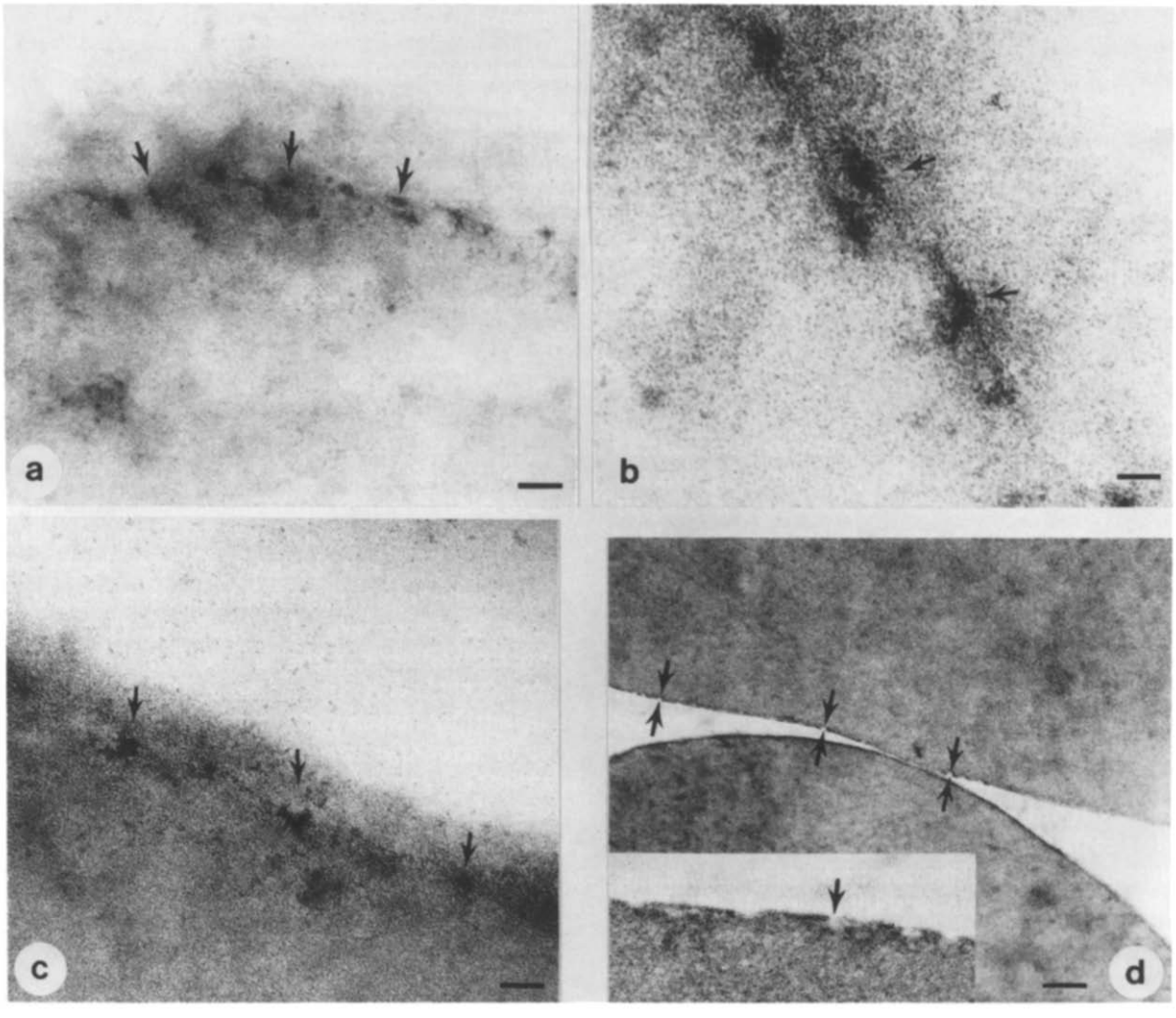

Fig.2. Electron micrographs showing association of 150 kDa dextrans (a,b,c) with the erythrocyte membrane. (a,b,c) Dextrans shown in close association with the red cell membrane (arrows). (b) A higher magnification of a section of a. (d) Membrane defects observed after incubation of red cells at pH 4.6, 1.5 min, 37°C in the absence of dextrans (arrows point to defects). Defects at higher magnification are shown in the inset. Bars represent 59 nm (a), 21 nm (b), 35 nm (c) and 170 nm (d).

tional diameter of such pores should be between 4 and 6 nm as suggested by the results in fig.1.

For FD70 the inhibition of hemolysis occurred at concentrations of 1–2 mg/ml (fig.1). It was shown that at this concentration, between 10^4 and 10^5 dextran molecules (77.6 kDa) are adsorbed on one human erythrocyte [1]. Our previous immunoelectron microscopy studies gave similar results for proteins: about 3×10^4 exogenous glycophorin epitopes were associated with one erythrocyte after the low-pH incubation under conditions of protection [9]. Thus, one possible explanation for the protective effect of proteins and dextrans is that they interact with the same sites on the RBC membrane, presumably the defects induced by low-pH conditions.

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